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<b>(54) Title:</b> MODIFIED POLYPEPTIDES WITH INCREASED BIOLOGICAL ACTIVITY  <b>(57) Abstract</b>  Modified polypeptides with increased biological activity exhibited as either increased potency or prolonged circulating half-life are disclosed with methods of preparing the modified polypeptides and methods of use.		

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MODIFIED POLYPEPTIDES WITH INCREASED  
BIOLOGICAL ACTIVITY

Background

Modification of naturally occurring polypeptides  
5 which have therapeutic value is often attempted in an  
effort to increase their biological activity. Several  
methods have been employed to increase the biological  
activity of therapeutic proteins. These methods often  
focus on increasing the size of the therapeutic agents.  
10 For example, the size of a protein can be increased  
through chemical conjugation with a reagent such as  
polyethylene glycol (PEG) (Knusli, C. et al., Brit. J.  
Haematol. 82:654-663 (1992)). This procedure, also known  
as "PEGylation", has been reported with several protein  
15 agents, first as a means to reduce antigenicity, but also  
as a way to increase biological activity.

Another method of increasing a protein's size is  
through chemical cross-linking with another protein. For  
example, to increase the antigenicity of a protein,  
20 chemical cross-linking agents are used to conjugate the  
immunogenic protein to a carrier molecule such as  
immunoglobulin or serum albumin.

However, the conjugation of chemical compounds or  
inert molecules to a polypeptide often results in a  
25 significant decrease of the overall biological activity,  
are of selected biological activity of the polypeptide,  
(Knusli, C., et al., Brit. J. Haematol., 82:654-663  
(1992)). These conjugations must be designed such that  
the resulting modified polypeptide remains therapeutically  
30 efficacious and retains the desired biological properties  
of the unmodified, wild type (i.e., naturally-occurring)  
polypeptide (Satake, R., et al., Biochem. Biophys. Acta.  
1038:125-129 (1990)).

Erythropoietin (EPO) is a glycoprotein hormone  
35 involved with the growth and development of mature red

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blood cells from erythrocyte precursor cells. It is a 166 amino acid polypeptide that exists naturally as a monomer. (Lin, F-K., et al. Proc. Natl. Acad. Sci. USA 82:7580-7584 (1985)).

5        Several forms of anemia, including those associated with renal failure, HIV infection, blood loss and chronic disease can be treated with this hematopoietic growth factor. Erythropoietin is typically administered by intravenous or subcutaneous injection three times weekly  
10 at a dose of approximately 25-100 U/kg. Though quite effective, this form of therapy is very expensive. Estimates for the treatment of chronic dialysis patients have ranged from \$8,000-10,000 per patient per year.

Another problem encountered in the practice of  
15 medicine when using injectable pharmaceuticals is the frequency at which those injections must be made in order to maintain a therapeutic level of the compound in the circulation. For example, erythropoietin has a relatively short plasma half-life (Spivak, J.L., and Hogans, B.B.,  
20 Blood, 73:90 (1989); McMahon, F.G., et al., Blood, 76:1718(1990)), therefore, therapeutic plasma levels are rapidly lost, and repeated intravenous administrations must be made. An alternative route of administration is subcutaneous injection. This route offers slower  
25 absorption from the site of administration, thus causing a sustained release effect. However, significantly lower plasma levels are achieved and, thus, a similar frequency of injection, as is required with intravenous administration, must be used to get a comparable  
30 therapeutic effect.

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Summary of the Invention

The present invention relates to modified polypeptides with increased biological activity, and methods of making these modified polypeptides. Increased  
5 biological activity is defined herein as a prolonged plasma half-life (i.e., a longer circulating half-life relative to the naturally occurring polypeptide), or higher potency (i.e., requiring a smaller quantity relative to the naturally occurring polypeptide to achieve  
10 a specified level of biological activity). Increased biological activity can also encompass a combination of the above-described activities, e.g., a modified polypeptide with higher potency that also exhibits a prolonged circulating half-life. In any case, because the  
15 polypeptides have increased biological activity, the frequency with which they must be administered is reduced, or the amount administered to achieve an effective dose is reduced. In any case, a reduced quantity of modified polypeptide would be necessary over the course of  
20 treatment than would be necessary if unmodified polypeptide were used.

Polypeptides encompassed by the present invention include, for example, hematopoietic growth factors such as colony stimulating factors (e.g., G-CSF and GM-CSF), the  
25 interleukins (e.g., IL-2 and IL-3), hormones such as basic fibroblast growth factor and glycoproteins such as human follicle stimulating hormone.

More specifically, the present invention relates to modified erythropoietin with increased biological  
30 activity, as defined above. The modified erythropoietin of the present invention comprises wild type

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erythropoietin that has been modified with a heterobifunctional cross-linking reagent. A heterobifunctional cross-linking reagent is defined herein as a reagent with two reactive groups that are capable of  
5 reacting with and forming links, or bridges, between the side chains of certain amino acids, between amino acids and carboxylic acid groups, or via carbohydrate moieties. In particular, the heterobifunctional cross-linking reagents used in the present invention contain either a  
10 cleavable disulfide bond group or a maleimido group.

The present invention also relates to multimeric erythropoietin comprising two, or more, erythropoietin molecules covalently linked together by one, or more, thioether bond(s). These erythropoietin multimers also  
15 exhibit increased biological activity. The present invention further relates to methods of producing the modified erythropoietin polypeptides with increased biological activity described herein, and to methods of their use.

20 The modification of wild type erythropoietin with a heterobifunctional cross-linking reagent containing a cleavable disulfide bond group resulted in a modified erythropoietin with increased potency relative to unmodified wild type erythropoietin. Importantly, the  
25 disulfide bond group can be reduced to a free sulfhydryl group. The availability of a free sulfhydryl group on the erythropoietin polypeptide permitted further modification of erythropoietin to produce multimeric erythropoietin with a prolonged circulating half-life relative to wild  
30 type erythropoietin. The production of multimeric erythropoietin was accomplished by a method of chemically

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cross-linking two, or more, modified erythropoietin polypeptides. Briefly, the method is as follows.

A first erythropoietin derivative was produced by reacting wild type erythropoietin with a  
5 heterobifunctional cross-linking reagent containing a cleavable disulfide bond group. The disulfide bond was reduced to produce erythropoietin containing a free sulfhydryl group. A second erythropoietin derivative was produced by reacting wild type erythropoietin with a  
10 heterobifunctional cross-linking reagent containing a maleimido group. The first and second erythropoietin derivatives were reacted together, thereby forming at least one thioether bond between the sulfhydryl and maleimido groups, thus forming a homodimer or homotrimer  
15 of erythropoietin. Surprisingly, these multimeric erythropoietin molecules exhibit biological activity comparable to wild type erythropoietin. More importantly, the erythropoietin dimers showed a significantly prolonged circulating half-life in vivo, relative to wild type  
20 erythropoietin.

Thus, as a result of the work presented herein, erythropoietin has now been modified to produce erythropoietin compositions which exhibit increased biological potency relative to wild type erythropoietin.  
25 Moreover, the modified erythropoietin of the present invention can be dimerized and trimerized with other modified erythropoietin molecules to produce multimeric erythropoietin molecules with prolonged in vivo circulating half-lives.

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Brief Description of the Figures

Figure 1A shows the chemical structure of SPDP.

Figure 1B shows the chemical structure of LC-SPDP.

Figure 1C shows the chemical structure of sulfo-LC  
5 SPDP.

Figure 2 shows the chemical structure of SMCC.

Figure 3 is a histogram depicting the biological activity of the fractions containing homotrimers, homodimers and monomers of erythropoietin collected after  
10 high pressure liquid chromatography (HPLC).

Figure 4 is a graphic representation of the results of a bioassay demonstrating the increased in vivo half-life of the erythropoietin dimer and monomer.

Detailed Description of the Invention

15 The present invention relates to modified polypeptides with increased biological activity, and methods of making and using these modified polypeptides. Polypeptides suitable for modification by the methods described herein are polypeptides, preferably monomeric  
20 polypeptides, which do not contain any free sulfhydryl groups. Polypeptides of special interest are those polypeptides which interact with a cellular receptor to initiate cellular signaling events, for example, insulin and erythropoietin. Polypeptides encompassed by the  
25 present invention are typically used as injectable therapeutic agents. If polypeptides with increased biological activity are used as injectable therapeutic agents, the frequency of administration of these polypeptides can be reduced.



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As described herein, these polypeptides can be modified to increase their biological activity relative to the biological activity of the naturally occurring polypeptides. Increased biological activity, is defined herein as a prolonged plasma half-life (i.e., a longer circulating half-life than the naturally occurring polypeptide), or higher potency (i.e., requiring a smaller quantity than the naturally occurring polypeptide to achieve a specified level of biological activity).

Increased biological activity, as used herein, can also encompass a combination of the above described activities. For example, a modified polypeptide with higher potency can also have an increased circulating half-life. In any case, because the polypeptides described herein have increased biological activity, the frequency with which they must be administered can be reduced.

The polypeptides encompassed by the present invention are modified with a heterobifunctional cross-linking reagent. The heterobifunctional cross-linking reagent can be attached to one, or more primary amine or amines, within the polypeptide. For example, the heterobifunctional cross-linking reagent can be attached to the amino acid residue, lysine or to the alpha amino terminus of erythropoietin. Alternatively, for glycoproteins, the heterobifunctional cross-linking reagent can be attached to one, or more carbohydrate moiety, or moieties, in an oligosaccharide chain on the polypeptide.

The heterobifunctional cross-linking reagent is generally selected from a group of cross-linking reagents containing either a cleavable disulfide bond group or a

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maleimido group. The addition of a disulfide bond group to a polypeptide also permits the design of a cross-linking strategy to produce multimeric polypeptides. The disulfide bond can be cleaved by reaction with a known  
5 reducing agent, for example, dithiothreitol (DTT) which reduces the disulfide bond in the cross-linking reagent to produce a modified polypeptide derivative containing a free sulfhydryl (SH) group.

A second polypeptide derivative, capable of reacting  
10 with a free sulfhydryl group, is then produced by attaching a heterobifunctional cross-linking reagent containing a maleimido group to the naturally occurring polypeptide. Again, the cross-linking reagent can be attached to primary amines or carbohydrate moieties in the  
15 polypeptide. The resulting polypeptide derivative containing a maleimido group is reacted with the polypeptide derivative containing a reactive sulfhydryl group resulting in a multimeric polypeptide molecule covalently linked together by at least one thioether bond  
20 formed between the SH group and the maleimido group.

Erythropoietin, a glycoprotein hormone involved with the growth and development of mature red blood cells from erythrocyte precursor cells, is a glycosylated polypeptide particularly suited for modification using the methods  
25 described herein. Erythropoietin is produced in the kidney in response to hypoxia (e.g., red blood cell loss due to anemia) and regulates red blood cell growth and differentiation through interaction with its cognate cellular receptor. Wild type erythropoietin is defined  
30 herein to include recombinant human erythropoietin (Powell, J.S., et al., Proc. Natl. Acad. Sci. USA,

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83:6465-6469 (1986)), or naturally occurring erythropoietin which has been purified from blood (Miyake, T., et al. J. Biol. Chem., 252:5558-5564 (1977)) or sheep plasma (Goldwasser, E., et al. Proc. Natl. Acad. Sci. U.S.A., 68:697-698 (1971)). Erythropoietin is a 166 amino acid polypeptide that exists naturally as a monomer. (Lin, F-K., et al. Proc. Natl. Acad. Sci. USA 82:7580-7584 (1985)). The predicted secondary structure of erythropoietin has been reported (McDonald, J.D., et al., 10 Mol. Cell. Biol., 6:842-848 (1986)).

It was noted from the structure of wild type erythropoietin that the polypeptide does not contain any free (reactive) sulfhydryl (SH) groups. (Boissel, J-P., et al., J. Biol. Chem. 268:15983-15993 (1993)). Free SH 15 groups are useful for preparing conjugated proteins, such as radiolabeled antibodies (U.S. Patent 4,659,839), or otherwise chemically modifying the polypeptide resulting in altered biological activity of a polypeptide. A free sulfhydryl group can also play a role in the binding of a 20 polypeptide to its cellular receptor. For example, the polypeptide hormone, insulin, is covalently linked to its cellular receptor via a disulfide exchange mechanism. (Clark, S. and Harrison, L. C., J. Biol. Chem., 258:11434-11437 (1983); Clark, S. and Harrison, L. C., J. Biol. 25 Chem., 257:12239-12344 (1982)). Thus, a free sulfhydryl group can be critical to the biological activity of a polypeptide. Accordingly, a scheme was devised to modify wild type erythropoietin to attach a free sulfhydryl group.

30 In one embodiment of the present invention, wild type erythropoietin was chemically modified by the covalent

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attachment of a heterobifunctional cross-linking reagent containing a cleavable disulfide bond group. The cross-linking reagent was attached to a primary amine in the erythropoietin polypeptide. The attachment of a  
5 heterobifunctional cross-linking reagent to wild type erythropoietin resulted in erythropoietin with increased potency relative to unmodified erythropoietin.

Specifically, three different heterobifunctional cross-linking reagents were used to produce modified  
10 erythropoietin with increased biological activity. These cross-linking reagents were attached to one, or more, primary amine or amines in the wild type erythropoietin. The cross-linking reagents were N-succinimidyl  
3-(2-pyridyldithio) propionate (SPDP), "long chain" N-  
15 succinimidyl 3-(2-pyridyldithio) propionate (LC-SPDP), wherein the length of the chain of SPDP is increased with additional methyl groups, and sulfonated "long-chain" N-succinimidyl 3-(2-pyridyldithio) propionate (sulfo-LC-SPDP) wherein LC-SPDP is sulfonated. SPDP (Figure 1A), LC-SPDP  
20 (Figure 1B) and sulfo-LC-SPDP (Figure 1C) are commercially available cross-linking agents (Pierce Chemical Co., Rockford Ill). SPDP, LC-SPDP and sulfo-LC-SPDP all contain an N-hydroxysuccinimidyl group to react with free amino groups. In addition, these reagents all contain a  
25 disulfide bond group that can be further modified to form a reactive sulfhydryl group.

Another heterobifunctional cross-linking reagent that can be used to modify wild type erythropoietin is a carbohydrate specific reagent that attaches to  
30 carbohydrate moieties of glycosylated polypeptides. This cross-linking reagent, 3-(2-pyridyldithio) propionyl

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hydrazide (PDPH), contains an oxidized carbohydrate specific hydrazide and also contains a cleavable disulfide bond group.

Wild type erythropoietin was modified with heterobifunctional cross-linking reagents SPDP, LC-SPDP and sulfo-LC-SPDP as described in detail in Example 1. Briefly, erythropoietin was incubated in the presence of specified concentrations of the chemical reagent N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) so as to achieve different molar ratios of SPDP:EPO in solution. The unmodified wild type erythropoietin and SPDP modified erythropoietin (SPDP-EPO) were bioassayed according to the method of Krystal, (Krystal, G., Exp. Hematol., 11:649-660 (1983)), which measures the effect of erythropoietin on erythropoiesis in intact mouse spleen cells. The results, shown in Table 1, demonstrate that SPDP-EPO exhibited an increased biological activity relative to the control wild type erythropoietin.

TABLE 120      SPECIFIC ACTIVITY OF SPDP-MODIFIED ERYTHROPOIETIN

Reaction Mixture, SPDP/EPO, mol/mol	Specific Activity U/mcg
0:1	200 ± 30
1:1	174 ± 20
3:1	340 ± 30

25      Erythropoietin modified with sulfo-LC-SPDP (sulfo LC-SPDP-EPO), which has the advantage of increased solubility

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in aqueous solutions, was also prepared as described in Example 1. Incubation of erythropoietin in the presence of sulfo-LC-SPDP at different molar ratios, followed by dialysis and biological assay revealed that sulfo-LC-SPDP  
5 modification of erythropoietin resulted in a 530% increase in potency over the activity of wild type erythropoietin, as shown in Table 2. Thus, the specific activity of the erythropoietin was increased from 170 U/mcg for the wild  
10 erythropoietin prepared in the presence of 10 fold molar excess of sulfo-LC-SPDP.

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TABLE 2

## SPECIFIC ACTIVITY OF SULFO-LC-SPDP MODIFIED ERYTHROPOIETIN

	Reaction Mixture, SULFO-LC-SPDP/EPO, mols/mol	Specific Activity $\mu$ /mcg
5	Experiment #1	
	0:1	170 $\pm$ 20
	5:1	220 $\pm$ 30
	10:1	900 $\pm$ 70
10	30:1	600 $\pm$ 50
	50:1	250 $\pm$ 30
	100:1	350 $\pm$ 40
	Experiment #2	
	0:1	200 $\pm$ 30
15	1:1	200 $\pm$ 40
	2:1	370 $\pm$ 40
	3:1	350 $\pm$ 40
	6:1	380 $\pm$ 40
	7:1	560 $\pm$ 50
20	10:1	900 $\pm$ 60

LC-SPDP EPO was also prepared as described in Example 1. Although the biological activity of this derivative was not evaluated, it is reasonable to believe that erythropoietin modified with LC-SPDP would also exhibit increased biological activity due to its close structural relationship to SPDP and sulfo-LC-SPDP.

The chemically modified erythropoietin derivatives described above, which contained a cleavable disulfide

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bond group, permitted the design of a strategy to cross-link erythropoietin to form EPO-EPO dimers and EPO-EPO-EPO trimers with increased biological activity. These homodimers (EPO-EPO) and homotrimers (EPO-EPO-EPO) are 5 "long-acting" erythropoietin proteins (also referred to herein as LA-EPOs). That is, these multimeric erythropoietin derivatives exhibit a prolonged circulating half-life relative to unmodified, erythropoietin.

The method of preparing multimeric erythropoietin 10 with increased biological activity is described in detail in Example 2. Although erythropoietin is used as the specific example, it is understood that the method described herein can be used to produce multimers (i.e., a polypeptide covalently cross-linked with one, or more, 15 identical polypeptides) of any suitable polypeptide.

Briefly, a first derivative of erythropoietin was prepared as described in Example 1, by reacting erythropoietin with the compound N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) to form SPDP-EPO. 20 This reaction introduced an external disulfide bond group into the erythropoietin molecule. To form a free (or reactive) sulfhydryl group, SPDP-EPO can be exposed to a reducing agent, known to those of skill in the art, to reduce the disulfide bond groups. As described in Example 25 2, SPDP-EPO was exposed to dithiothreitol (DTT), which reduces the disulfide bond in the SPDP moiety to produce an erythropoietin molecule containing free SH groups, also referred to herein as SH-EPO.

A second erythropoietin derivative was produced by 30 reacting erythropoietin with succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate, also



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known as SMCC (Figure 2) to form SMCC-EPO. This reagent has an N-hydroxy succinimidyl (NHS) group at one end and a maleimido group at the other. The NHS group of SMCC reacts with free amino groups in erythropoietin resulting in the formation of SMCC-EPO. The maleimido group of SMCC, now pointing outward from the SMCC-EPO derivative, reacts with free sulfhydryl groups found on SH-EPO. Therefore, when SH-EPO and SMCC-EPO are mixed together in solution, the reactive groups combine resulting in the formation of an EPO-EPO dimer, (i.e., one SH-EPO with one SMCC-EPO) or an EPO-EPO-EPO trimer (i.e., one SMCC-EPO with two SH-EPOs, or two SMCC-EPOs with one SH-EPO) in which the modified erythropoietin polypeptides are covalently linked by at least one thioether bond (e.g., one thioether bond in dimerized EPO and two thioether bonds in trimerized EPO). It is interesting to note that SMCC-EPO, when tested in the Krystal bioassay, did not exhibit any increased biological activity relative to unmodified erythropoietin.

Alternatively, a heterobifunctional cross-linking reagent which contains a maleimido group to attach to carbohydrate moieties such as 4-(4-N-maleimidophenyl) butyric acid hydrazide-HCl (MPBH) and 4-(N-maleimidomethyl) cyclohexane-1-carboxyl-hydrazide-HCl, can be used.

The first and second erythropoietin derivatives were reacted together as described in detail in Example 2. The reaction resulted in the formation of multimeric erythropoietin, as well as unreacted monomeric erythropoietin derivatives, which can be separated by high pressure liquid chromatography (HPLC), as described in

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Example 2. The erythropoietin dimers comprised two erythropoietin polypeptides linked by one or more thioether bonds. The erythropoietin trimers comprised three erythropoietin polypeptides, also linked by 5 thioether bonds. The trimer can comprise two erythropoietin polypeptides, each containing a free sulfhydryl group which is linked with a third erythropoietin polypeptide containing two or more, maleimido groups. Alternatively, the erythropoietin 10 trimer can comprise one erythropoietin polypeptide containing two, or more, free sulfhydryl groups which is linked with two erythropoietin polypeptides, each containing a maleimido group. The presence of EPO, EPO-EPO dimers and EPO-EPO-EPO trimers was confirmed by 15 Western blot analysis using antibodies specific for erythropoietin as described in Sytkowski, A.J., and Fisher, J.W., J. Biol. Chem., 260:14727-14731 (1985).

Although the monomeric erythropoietin retained its biological activity, the erythropoietin dimers and trimers 20 prepared under the conditions described in Example 2, with SH-EPO, did not exhibit biological activity when tested in the Krystal bioassay. Therefore, a second cross-linking protocol was designed in which a second type of SH-EPO derivative was prepared using sulfo-LC-SPDP. This agent 25 functions similarly to SPDP as outlined above, however, it contains a spacer arm of several angstroms in length (e.g., wherein the number of CH<sub>2</sub> groups in the linear portion of the molecule is increased) resulting in increased physical separation of the species attached to 30 its reactive ends. In particular, sulfo-LC-SPDP contains five methyl groups within the linear chain of the

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molecule, and is also sulfated to increase its aqueous solubility.

Multimeric erythropoietin produced using sulfo-LC-SPDP-EPO (SH-LC-EPO) as the first erythropoietin derivative was prepared, and separated by HPLC as described in detail in Example 2. HPLC fractions containing the trimers, dimers and monomers were tested in the Krystal bioassay for biological activity. Importantly, all three of these species, monomers, dimers, and trimers exhibited biological activity in the Krystal assay. (See Figure 3).

The circulating half-life in vivo of erythropoietin homodimers was determined as described in detail in Example 4. Monomeric and dimeric erythropoietin was injected into rabbits, and blood samples were analyzed at 5 minutes and 2, 4, 6, 9, and 24 hours after injection. As shown in Figure 4, the biological activity of dimerized erythropoietin, as measured in the Krystal assay, was still evident 24 hours after the initial injection, whereas the biological activity of monomeric erythropoietin dropped off significantly earlier. Thus, the circulating half-life of dimerized erythropoietin was more than three times longer than wild type erythropoietin. The prolonged circulating half-life of the EPO dimer may be due to its increased size relative to monomeric erythropoietin, which would hinder its excretion from the body through the kidney. Although the erythropoietin trimers were not assayed at this time, it is reasonable to predict that an EPO homotrimer would exhibit similar, or even longer circulatory half-life as the homodimers because a trimer has even greater size than

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a dimer. These erythropoietin dimers and trimers are also referred to herein as long-acting erythropoietins (LA-EPOs).

Thus, as a result of the work described herein,  
5 modified erythropoietin polypeptides are provided which exhibit increased biological activity. Erythropoietin modified with a heterobifunctional cross-linking reagent containing a cleavable disulfide bond group exhibited a 530% increase in biological activity relative to wild type  
10 erythropoietin. This increase in biological activity indicates that an effective amount of modified erythropoietin is substantially less than a comparable effective amount of wild type erythropoietin. The effective amount of modified erythropoietin is defined  
15 herein as the amount of erythropoietin required to elicit an erythropoietic response, as indicated by increased growth and/or differentiation of erythrocytic precursor cells. For example, if the typical effective dose of erythropoietin used therapeutically is 25 U/kg, then an  
20 effective dose of modified erythropoietin can reasonably be as low as 5.0 U/kg to achieve the same effect.

Alternatively, the effective amount of multimeric erythropoietin described herein, with a prolonged circulating half-life, will require less frequent  
25 administration than an equivalent amount of wild type erythropoietin. For example, if an effective dose of erythropoietin is typically administered 3 times a week, multimeric erythropoietin with increased biological activity will only need to be administered once a week.  
30 In either case, a reduced quantity of erythropoietin modified with a heterobifunctional cross-linking reagent,

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or multimeric erythropoietin, will be required over the course of treatment than is necessary if wild type erythropoietin is used.

The modified erythropoietin with increased biological  
5 activity described herein can be used in place of wild type erythropoietin whenever treatment with erythropoietin is called for. For example, modified erythropoietin can be used for treatment in an individual experiencing anemia associated with renal failure, chronic disease, HIV  
10 infection, blood loss or cancer.

Erythropoietin is generally administered to humans. Effective treatment with erythropoietin requires maintaining a therapeutic blood level. This can be done by continuous administration, that is, by continuous  
15 intravenous injections, by discreet intravenous injections, or by subcutaneous injection. The modified erythropoietin of this invention can be employed in admixture with conventional excipients, i.e., pharmaceutically acceptable organic or inorganic carrier  
20 substances suitable for parenteral administration that do not deleteriously react with the active derivatives.

Suitable pharmaceutically acceptable carriers include, but are not limited to, water, salt solutions, alcohols, gum arabic, vegetable oils, benzyl alcohols,  
25 polyethylene glycols, gelatine, carbohydrates such as lactose, amylose or starch, magnesium stearate, talc, silicic acid, viscous paraffin, perfume oil, fatty acid esters, hydroxymethylcellulose, polyvinyl pyrrolidone, etc. For parenteral application, particularly suitable are  
30 injectable, sterile solutions, preferably oily or aqueous

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solutions, as well as suspensions, emulsions, or implants, including suppositories.

It will be appreciated that the actual preferred amounts of active compound in a specific case will vary according to the specific compound being utilized, the particular compositions formulated, the mode of application, the particular situs of application, and the organism being treated. Dosages for a given recipient will be determined on the basis of individual characteristics, such as body size, weight, age and the type and severity of the condition being treated.

In addition, the modified erythropoietin of the present invention, with increased biological activity, can be used in any in vitro application in place of wild type erythropoietin. For example, modified erythropoietin can be used in studies of erythropoietin receptor activity. It will again be appreciated that the amount of modified erythropoietin with increased biological activity needed to achieve desired results, (e.g., increased hemoglobinization of red blood cell precursor cells) will be substantially less than the amount of wild type erythropoietin required to achieve those desired results.

The present invention will now be illustrated by the following examples, which are not intended to be limiting in any way.

Example 1: SPDP-EPO Derivative with Higher Potency

Three different heterobifunctional cross-linking reagents containing cleavable disulfide bond groups have been used to produce erythropoietin derivatives with increased biological activity. These agents are N-

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succinimidyl 3-(2-pyridyldithio) propionate (SPDP), "long-chain" N-succinimidyl 3-(2-pyridyldithio) propionate, wherein the length of the chain of SPDP is increased with additional methyl groups (LC-SPDP), and sulfonated  
5 "long-chain" N-succinimidyl 3-(2-pyridyldithio) propionate (sulfo-LC-SPDP). Modified erythropoietin polypeptides were prepared as follows.

Recombinant human erythropoietin was produced by expression of the human erythropoietin gene in stably  
10 transfected BHK (baby hamster kidney) cells (Powell, J.S. et al., Proc. Nat. Sci. Acad. USA., 83:6465-6469 (1986) and purified using standard laboratory techniques. The purified protein was then incubated in the presence of specified concentrations of the chemical reagent  
15 N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP), dissolved in dimethyl sulfoxide, so as to achieve molar ratios of 0:1, 1:1 and 3:1 (SPDP:EPO) in solution. After incubation overnight at room temperature, the solutions were dialyzed against phosphate buffered saline to remove  
20 unreacted SPDP.

The wild type erythropoietin and modified erythropoietin (SPDP-EPO) samples were evaluated for biological activity according to the method of Krystal. (Krystal, G., Exp. Hematol., 11:649-660 (1983)). Briefly,  
25 the bioassay of Krystal measures the effect of erythropoietin on intact mouse spleen cells. Mice are treated with phenylhydrazine to stimulate production of red blood cell precursor cells in the spleen. After treatment, the spleens are removed, intact spleen cells  
30 are carefully isolated and incubated with various amounts of wild type erythropoietin or the modified erythropoietin

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described herein. After an overnight incubation,  $^3\text{H}$  thymidine is added and its incorporation into cellular DNA is measured. The amount of  $^3\text{H}$  thymidine incorporation is indicative of erythropoietin-stimulated production of red blood cells via interaction of erythropoietin with its cellular receptor. The results demonstrate that SPDP-EPO exhibited an increased biological activity relative to the wild type erythropoietin, and that this increase in activity was proportional to the molar ratio of SPDP:EPO in the reaction mixture.

Additionally, wild type erythropoietin was modified using sulfo-LC-SPDP, a compound which has the advantage of increased solubility in aqueous solutions. Incubation of erythropoietin in the presence of sulfo-LC-SPDP at the previously described molar ratios followed by dialysis and biological assay revealed that sulfo-LC-SPDP modification of erythropoietin resulted in an increase in potency of approximately 530%. The specific activity of the erythropoietin was increased from 170 U/mcg for the nonderivatized material to 900 U/mcg for the material derivatized in the presence of 10 fold molar excess of sulfo-LC-SPDP.

Example 2: Long-Acting Multimeric Erythropoietin Derivatives

To prepare the first SH-EPO derivative, 50 ug of human erythropoietin obtained as described in Example 1, was incubated in the presence of five-fold molar excess of N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) obtained from Pierce Chemical Company. After incubation at room temperature for sixteen hours, the solution was



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dialyzed against phosphate buffered saline. The modified erythropoietin was then exposed to 1mM DTT to reduce the disulfide bond in SPDP resulting in one, or more, free sulfhydryl group(s) on the erythropoietin molecule.

5       The second erythropoietin derivative, SMCC-EPO, was prepared as follows. A second 50 ug portion of human erythropoietin was incubated in the presence of five-fold molar excess of succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC). After a sixteen hour  
10 incubation at room temperature, the solution was dialyzed against phosphate buffered saline.

The SH-EPO and SMCC-EPO were mixed together in phosphate buffered saline (20 mM sodium phosphate, 150 mM sodium chloride, pH 7.4) at room temperature for 90  
15 minutes, and dialyzed against PBS. The mixture was then subjected to size exclusion HPLC chromatography on TSK 250, in PBS, room temperature, at 1 ml/min. The polypeptides were subjected to SDS polyacrylamide gel electrophoresis, electrophoretic transfer to  
20 nitrocellulose, and Western blotting using anti-erythropoietin antibodies according to Sytkowski, A. J., and Fisher, J. W., J. Biol. Chem., 260:14727-14731 (1985). The results showed that the protocol succeeded in the formation of two higher molecular weight species of  
25 erythropoietin corresponding to erythropoietin dimers and trimers. However, upon assay in the Krystal bioassay, the erythropoietin dimers and trimers produced with SPDP-SH-EPO did not exhibit any biological activity.

Thus, the protocol was revised to use LC-SPDP-EPO as  
30 the first derivative, 50 ug of recombinant human erythropoietin was incubated in the presence of three-fold

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molar excess of LC-SPDP for sixteen hours at room temperature. The material was then dialyzed and treated with 1 mM DTT resulting in SH-LC-EPO. SMCC-EPO was prepared as described above.

5        These two species were mixed together in solution and the mixture was subjected to size exclusion HPLC on TSK3000SW. Three erythropoietin protein species were detected with elution times of 10.2, 9.1, and 7.2 minutes respectively. The 10.2 minute elution time was known from  
10 previous experiments to be that of wild type erythropoietin monomer. Therefore, the more rapid elution times of 9.1 and 7.2 minutes corresponded to dimers and trimers, respectively. The fractions containing the erythropoietin dimers and trimers were collected and  
15 assayed in the Krystal bioassay. Importantly, as shown in Figure 3, upon testing in the Krystal bioassay, the erythropoietin homodimers and homotrimers exhibited biological activity.

Example 3: In Vivo Testing of Multimeric Erythropoietin  
20 Derivatives

A group of New Zealand white rabbits were injected intravenously either with wild type monomeric erythropoietin or with dimerized LA-EPO at 0.4 mg/ml in PBS. Blood samples were obtained at 5 minutes and 2, 4,  
25 6, 9, and 24 hours and measured the circulating erythropoietin by the Krystal in vitro biologic assay. The results shown in Figure 4 indicate that the in vivo half-life for monomeric wild type erythropoietin was approximately seven hours, as expected from previously  
30 published reports. The in vivo half-life of LA-EPO

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however, was prolonged beyond the twenty-four hour period of the experiment as shown in Figure 4.

#### EQUIVALENTS

Those skilled in the art will recognize, or be able  
5 to ascertain using no more than routine experimentation,  
many equivalents to specific embodiments of the invention  
described specifically herein. Such equivalents are  
intended to be encompassed in the scope of the following  
claims.

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CLAIMS

The invention claimed is:

1. Modified erythropoietin comprising erythropoietin having a heterobifunctional cross-linking reagent containing a cleavable disulfide bond group.
2. Modified erythropoietin of Claim 1 wherein the heterobifunctional cross-linking reagent is attached to one, or more, primary amine or amines in the erythropoietin molecule.
3. Modified erythropoietin of Claim 2 wherein the heterobifunctional cross-linking reagent is selected from the group consisting of: N-succinimidyl 3-(2 pyridyldithio) propionate, succinimidyl 6-[3-(2 pyridyldithio) propionamido] hexanoate, and sulfosuccinimidyl 6-[3-(2 pyridyldithio) propionamido] hexanoate.
4. Modified erythropoietin of Claim 1 wherein the erythropoietin is glycosylated erythropoietin and the heterobifunctional cross-linking reagent is attached to one, or more, carbohydrate moiety or moieties in the glycosylated erythropoietin.
5. Modified erythropoietin of Claim 4 wherein the heterobifunctional cross-linking reagent is 3-(2-pyridyldithio) propionyl hydrazide.

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6. Modified erythropoietin comprising erythropoietin having a heterobifunctional cross-linking reagent containing a free sulfhydryl group.
7. A pharmaceutical composition comprising an effective amount of modified erythropoietin comprising erythropoietin having a heterobifunctional cross-linking reagent containing a cleavable disulfide bond group and a pharmaceutically acceptable carrier.
8. A pharmaceutical composition comprising an effective amount of modified erythropoietin comprising erythropoietin having a heterobifunctional cross-linking reagent containing a free sulfhydryl group and a pharmaceutically acceptable carrier.
9. A method of treating anemia in an individual comprising administering to the individual a therapeutically effective amount of modified erythropoietin comprising erythropoietin having a heterobifunctional cross-linking reagent containing a cleavable disulfide bond group.
10. A method of treating anemia in an individual comprising administering to the individual a therapeutically effective amount of modified erythropoietin comprising erythropoietin having a heterobifunctional cross-linking reagent containing a free sulfhydryl group.

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11. Modified erythropoietin comprising erythropoietin having a heterobifunctional cross-linking reagent containing a maleimido group.
- 5 12. Modified erythropoietin of Claim 11 wherein the heterobifunctional cross-linking reagent is attached to one, or more, primary amine or amines in the erythropoietin molecule.
13. Modified erythropoietin of Claim 12 wherein the heterobifunctional cross-linking reagent is  
10 sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate.
14. Modified erythropoietin of Claim 11 wherein the erythropoietin is glycosylated erythropoietin and the heterobifunctional cross-linking reagent is attached  
15 to one, or more, carbohydrate moiety or moieties in the glycosylated erythropoietin.
15. Modified erythropoietin of Claim 14 wherein the heterobifunctional cross-linking reagent is selected from the group consisting of: 4-(4-N-maleimidophenyl)  
20 butyric acid hydrazide-HCl (MPBH) and 4-(N-maleimidomethyl) cyclohexane-1-carboxyl-hydrazide-HCl.
16. Modified erythropoietin comprising two or more erythropoietin polypeptides covalently linked by at  
25 least one thioether bond.

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17. Modified erythropoietin comprising two erythropoietin polypeptides covalently linked by at least one thioether bond, wherein the first erythropoietin polypeptide comprises erythropoietin with a  
5 heterobifunctional cross-linking reagent containing a free sulfhydryl group and the second erythropoietin polypeptide comprises erythropoietin with a heterobifunctional cross-linking reagent containing a maleimido group, and the thioether bond forms between  
10 the free sulfhydryl group of the first erythropoietin polypeptide and the maleimido group of the second erythropoietin polypeptide.
18. Modified erythropoietin comprising three erythropoietin polypeptides covalently linked by  
15 thioether bonds, wherein the first and second erythropoietin polypeptides comprise erythropoietin with a heterobifunctional cross-linking reagent containing a free sulfhydryl group and the third erythropoietin polypeptide comprises erythropoietin  
20 with a heterobifunctional cross-linking reagent containing two, or more, maleimido groups, and the thioether bonds form between the free sulfhydryl group of the first and second erythropoietin polypeptides and the maleimido groups of the third  
25 erythropoietin polypeptide.
19. Modified erythropoietin comprising three erythropoietin polypeptides covalently linked by thioether bonds, wherein the first erythropoietin

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- polypeptide comprises erythropoietin with a heterobifunctional cross-linking reagent containing two, or more, free sulfhydryl groups and the second and third erythropoietin polypeptides comprise erythropoietin with a heterobifunctional cross-linking reagent containing a maleimido group, and the thioether bond forms between the free sulfhydryl groups of the first erythropoietin polypeptide and the maleimido group of the second and third erythropoietin polypeptides.
20. Modified erythropoietin of Claim 17 wherein the heterobifunctional cross-linking reagents are attached to one, or more, primary amine or amines in the erythropoietin polypeptides.
21. Modified erythropoietin of Claim 17 wherein the heterobifunctional cross-linking reagents are attached to one, or more, carbohydrate moiety or moieties of the erythropoietin polypeptides.
22. A method of preparing modified polypeptides comprising two or more identical polypeptides covalently linked by at least one thioether bond, comprising the steps consisting of:
- a) preparing a first polypeptide derivative by reacting a polypeptide with a heterobifunctional cross-linking reagent containing a cleavable disulfide bond group, under conditions sufficient to introduce the cleavable disulfide bond group into the polypeptide molecule,



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- thereby producing a first polypeptide derivative containing a cleavable disulfide bond;
- b) cleaving the disulfide bond contained in the first polypeptide derivative to produce a free  
5       sulfhydryl group, thereby producing a first polypeptide derivative containing a free sulfhydryl group;
- c) preparing a second polypeptide derivative by reacting a polypeptide with a heterobifunctional  
10       cross-linking reagent containing a maleimido group, under conditions sufficient to introduce the maleimido group into the polypeptide molecule, thereby producing a second polypeptide derivative containing a maleimide group; and
- 15       d) reacting the first polypeptide derivative containing a free sulfhydryl group with the second polypeptide derivative containing a maleimido group, under conditions sufficient to  
20       form a thioether bond between the free sulfhydryl group and the maleimido group resulting in the cross-linking of the polypeptide derivatives, thereby producing a modified polypeptide.
23. A method of Claim 22 wherein the heterobifunctional  
25       cross-linking reagents of step a) and step b) react with one, or more, primary amine or amines in the polypeptide.
24. A method of Claim 23 wherein the heterobifunctional cross-linking reagent of step a) is selected from the

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- group consisting of: N-succinimidyl 3-(2  
pyridyldithio) propionate, succinimidyl 6-[3-(2  
pyridyldithio) propionamido] hexanoate, and  
sulfosuccinimidyl 6-[3-(2 pyridyldithio)  
5 propionamido] hexanoate.
25. A method of Claim 23 wherein the cross-linking  
reagent of step b) is sulfosuccinimidyl 4-(N-  
maleimidomethyl)cyclohexane-1-carboxylate.
26. A method of Claim 22 wherein the polypeptide is  
10 glycosylated and the heterobifunctional cross-linking  
reagents of step a) and step b) react with one, or  
more, carbohydrate moiety or moieties in the  
glycosylated polypeptide.
27. A method of Claim 26 wherein the cleavable  
15 heterobifunctional cross-linking reagent of step a)  
is 3-(2-pyridyldithio) propionyl hydrazine.
28. A method of Claim 26 wherein the heterobifunctional  
cross-linking reagent of step b) is selected from the  
group consisting of: 4-(4-maleimidophenyl) butyric  
20 acid hydrazine-HCl and 4-(4-maleimidomethyl)  
cyclohexane-1-carboxyl hydrazine-HCl.
29. A modified polypeptide comprising two or more  
identical polypeptides covalently linked by at least  
one thioether bond produced by the method of Claim  
25 22.

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30. A method of preparing modified erythropoietin comprising two or more erythropoietin polypeptides covalently linked by at least one thioether bond, comprising the steps consisting of:
- 5 a) preparing a first erythropoietin derivative by reacting erythropoietin with a heterobifunctional cross-linking reagent containing a cleavable disulfide bond group, under conditions sufficient to introduce the  
10 cleavable disulfide bond group into the erythropoietin molecule, thereby producing a first erythropoietin derivative containing a cleavable disulfide bond;
  - 15 b) cleaving the disulfide bond group contained in the first erythropoietin derivative, thereby producing a first erythropoietin derivative containing a free sulfhydryl group;
  - 20 c) preparing a second erythropoietin derivative by reacting erythropoietin with a heterobifunctional cross-linking reagent containing a maleimide group, under conditions sufficient to introduce the maleimido group into the erythropoietin molecule, thereby producing a second erythropoietin derivative containing a  
25 maleimido group; and
  - 30 d) reacting the first erythropoietin derivative containing a free sulfhydryl group with the second erythropoietin derivative containing a maleimide group, under conditions sufficient to form a thioether bond between the free sulfhydryl group and the maleimido group

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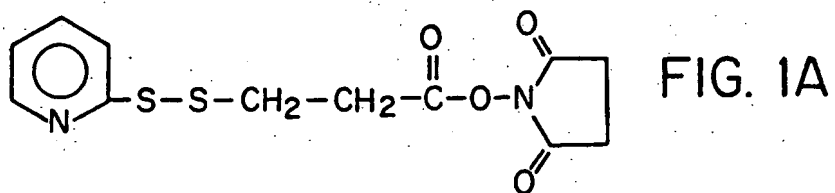
resulting in the cross-linking of the erythropoietin derivatives, thereby producing modified erythropoietin.

31. A method of Claim 30 wherein the heterobifunctional cross-linking reagents of step a) and step b) react with one, or more, primary amine or amines in the erythropoietin molecule.
32. A method of Claim 31 wherein the heterobifunctional cross-linking reagent of step a) is selected from the group consisting of: N-succinimidyl 3-(2 pyridyldithio) propionate, succinimidyl 6-[3-(2 pyridyldithio) propionamido] hexanoate, and sulfosuccinimidyl 6-[3-(2 pyridyldithio) propionamido] hexanoate.
33. A method of Claim 31 wherein the cross-linking reagent of step b) is sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate.
34. A method of Claim 30 wherein the erythropoietin is glycosylated erythropoietin and the heterobifunctional cross-linking reagents of step a) and step b) react with one, or more, carbohydrate moiety or moieties in the glycosylated erythropoietin.
35. A method of Claim 34 wherein the cleavable heterobifunctional cross-linking reagent of step a) is 3-(2-pyridyldithio) propionyl hydrazine.

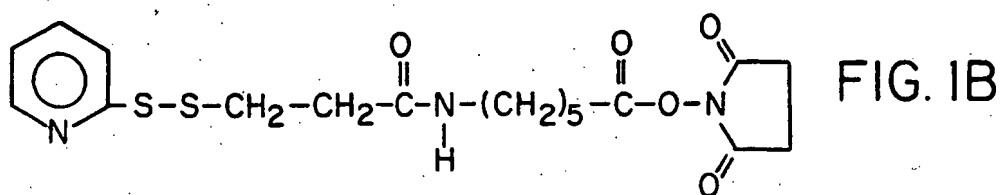
-35-

36. A method of Claim 34 wherein the heterobifunctional cross-linking reagent of step b) is selected from the group consisting of: 4-(4-maleimidophenyl) butyric acid hydrazine-HCl and 4-(4-maleimidomethyl) cyclohexane-1-carboxyl hydrazine-HCl.
37. A modified erythropoietin comprising two or more erythropoietin molecules covalently linked by at least one thioether bond produced by the method of Claim 30.
38. A pharmaceutical composition containing an effective amount of modified erythropoietin comprising two or more erythropoietin molecules covalently linked by at least one thioether bond and a pharmaceutically acceptable carrier.
39. A method of treating anemia in an individual comprising administering to the individual a therapeutically effective amount of modified erythropoietin comprising two or more erythropoietin molecules covalently linked by at least one thioether bond.

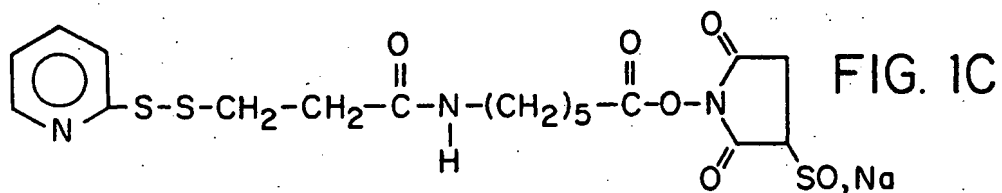
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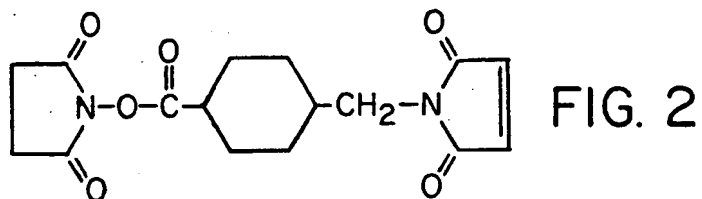
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6.8 Å



LC-SPDP  
M.W. 425.52



Sulfo-LC-SPDP  
M.W. 527.56



SMCC  
M.W. 334.33  
11.6 Å

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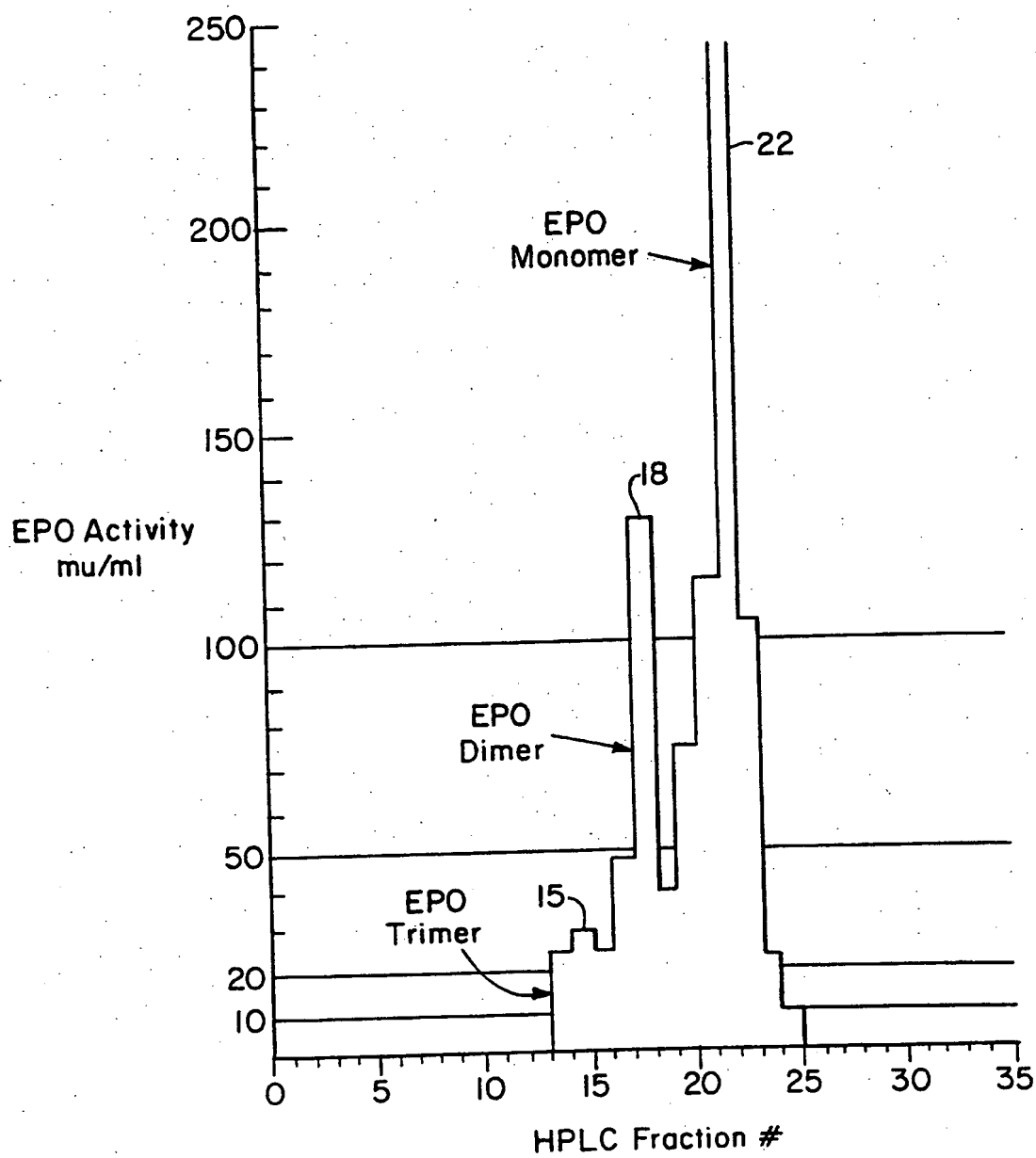


FIG. 3

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## PHARMACOKINETICS OF EPO

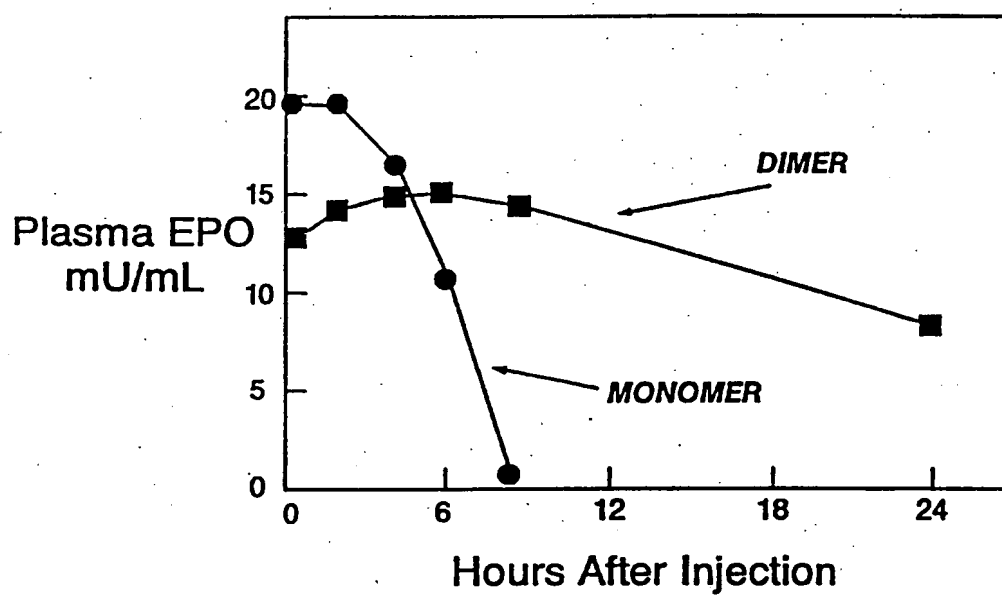


Figure 4



# INTERNATIONAL SEARCH REPORT

Intern al Application No

PCT/US 95/03242

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C07K14/505

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PROTEIN SCIENCE, vol. 2, 1993 USA, pages 1441-51, M.HANIU ET AL. 'Recombinant human erythropoietin (rHuEPO): Crosslinking with disuccinimidyl esters and identification of the interfacing domains in EPO' see the whole document ---	1,2,4-39
Y	PIERCE 'Pierce 1989 Handbook & General Catalog', PIERCE EUROPE B.V. , NL see page 286 - page 311 --- -/--	1,2,4-39

☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

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Date of the actual completion of the international search

4 July 1995

Date of mailing of the international search report

04. 08. 95

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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 95/03242

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J.BIOL.CHEM, vol. 266, no. 34, 1991 pages 23380-23385, P.MAYEUX ET AL. 'Structure of the Murine Erythropoietin Complex' see the whole document ---	22-29
Y	BIOCHIM.BIOPHYS.ACTA, vol. 761, 1983 pages 152-62, S.M.JUNG ET AL. 'Crosslinking of Platelet Glycoprotein 1b by N-Succinimidyl(4-Azidophenyldithio)Propion ate and 3,3'-Dithiobis(sulfosuccinimidyl Propionate)' see the whole document -----	22-29